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## Rapid induction of apoptosis by deregulated uptake of polyamine analogues

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Treatment of Chinese hamster ovary cells with  $\alpha$ -difluoromethylornithine for 3 days, followed by exposure to cycloheximide, led to an unregulated, rapid and massive accumulation of polyamine analogues. This accumulation led to cell death by apoptosis within a few hours. Clear evidence of DNA fragmentation was seen in response to both N-terminally ethylated polyamines and to polyamines containing methyl groups on the terminal carbon atoms. Programmed cell death was induced within 2–4 h of exposure to  $1\ \mu\text{M}$  or higher concentrations of  $N^1,N^{11}$ -bis(ethyl)norspermine. The presence of cycloheximide increased the uptake of the polyamine analogues and therefore led to cell death at lower analogue concentrations, but it was not essential for the induction of apoptosis, since similar effects were seen

when the protein synthesis inhibitor was omitted and the concentration of  $N^1,N^{11}$ -bis(ethyl)norspermine was increased to  $5\ \mu\text{M}$  or more. The induction of apoptosis was blocked both by the addition of the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, or by the addition of the polyamine oxidase inhibitor  $N^1$ -methyl- $N^2$ -(2,3-butadienyl)butane-1,4-diamine (MDL 72,527). These experiments provide evidence to support the concepts that: (1) polyamines or their oxidation products may be initiators of programmed cell death; (2) regulation of polyamine biosynthesis and uptake prevents the accumulation of toxic levels of polyamines; and (3) the anti-neoplastic effects of bis(ethyl) polyamine analogues may be due to the induction of apoptosis in sensitive tumour cells.

### INTRODUCTION

It is well known that the enzymes controlling the polyamine content of mammalian cells are very highly regulated, although the physiological reasons for this regulation are not well understood [1,2]. An increase in polyamine content causes a reduced rate of synthesis of the key biosynthetic enzymes, ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase, and an increased rate of degradation of these proteins. Furthermore, polyamine uptake is repressed by a high intracellular content [3] and the enzyme spermidine/spermine- $N^1$ -acetyltransferase (SSAT) is induced by polyamines [4]. The acetylation of polyamines brought about by this enzyme not only directly reduces their levels but also facilitates their degradation by FAD-dependent polyamine oxidase (PAO) and their excretion from the cell. It has been suggested that these factors and polyamine sequestration act to prevent the cellular polyamine content from reaching toxic levels [5,6]. Toxic effects of polyamines have been reported in many studies ([7–11] and references therein), although the mechanism by which toxic effects occur remains unclear and may be variable, involving oxidation products in some cases but not in others.

There has been considerable recent interest in the development of polyamine analogues as therapeutic agents. A series of polyamines with alkyl substituents on the terminal nitrogen atoms have been synthesized, and many of these have been shown to have potential as anti-tumour agents [12–15]. The mechanism by which these analogues cause the death of tumour cells is also not fully understood, and it is not clear whether the analogues act as polyamine agonists or antagonists in bringing about their toxic effects on the cell.

One possibility is that they act by means of reducing the intracellular polyamine content below levels compatible with cell survival. In support of this concept, it has been shown that the analogues do cause an inhibition of normal polyamine synthesis by reducing ODC and *S*-adenosylmethionine decarboxylase activities [16]. The analogues bring about this effect by mimicking the action of the natural polyamines on the synthesis and degradation of these enzymes. A second action of the polyamine analogues is to cause a massive induction of SSAT [1,2,4]. Some of these compounds are the best known inducers of SSAT and they cause an increase in SSAT mRNA level, its translation into protein and the stability of the SSAT protein. This combined with the fact that they are not themselves substrates for acetylation by SSAT, and are therefore not depleted by SSAT induction, causes SSAT to rise to very high levels. The combination of a reduction of polyamine synthesis and an increase in polyamine degradation and excretion produces a rapid and profound reduction in intracellular polyamine levels, which is exacerbated by the fact that the polyamine analogues are substrates for the polyamine transport system and their competition for this carrier prevents uptake of extracellular polyamines.

On the other hand, the growth inhibitory effects of polyamine analogues do not correlate particularly well with their abilities to induce SSAT or with the speed of polyamine depletion, and a better correlation was seen with the intracellular analogue concentration itself [17–19]. It was therefore suggested that the accumulation of the polyamine analogues and their binding to sites normally occupied by polyamines may cause cytotoxicity. The depletion of the normal polyamines could enhance this effect by facilitating the binding of the analogues to the sites. Other

Abbreviations used: ODC, ornithine decarboxylase; SSAT, spermidine/spermine- $N^1$ -acetyltransferase; PAO, FAD-dependent polyamine oxidase; BE-3-3-3, 3,7,11,15-tetra-azaheptadecane [also known as  $N^1,N^{11}$ -bis(ethyl)norspermine]; BE-3-4-3, 3,7,12,16-tetra-azaocadecane [also known as  $N^1,N^{12}$ -bis(ethyl)spermine]; BE-3-7-3,  $N,N$ -bis[3-(ethylamino)-propyl]-1,7-heptane diamine; BE-4-4-4, 3,8,13,18-tetra-azaeicosane; BE-4-4-4-1, 1,19-bis(ethylamino)-5,10,15-triazanonadecane; DFMO,  $\alpha$ -difluoromethylornithine; MDL 72,527,  $N^1$ -methyl- $N^2$ -(2,3-butadienyl)butane-1,4-diamine; 1-Me-SPD, 1-methylspermidine; 1,12-Me<sub>2</sub>-SPM, 1,12-dimethylspermine; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; TLCK, 1-tosylamido-2-phenylethylchloromethyl ketone.

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possibilities, including the interference with the formation of hypusine in the putative initiation factor eIF-5A, have also been proposed [9].

Further information on the mechanism by which the polyamine analogues exert their effects would clearly be valuable in aiding in the design and therapeutic use of such compounds. In the present studies, we have taken advantage of the recent findings that polyamine transport is repressed by the action of a highly labile protein termed antizyme. Antizyme was first described as an inhibitor of ODC but was subsequently shown to function by binding to the ODC monomer and facilitating its degradation by the proteasome [20]. More recently, it was found that antizyme, which is induced by polyamines, also acts to block the transport system [21–23]. The use of an inhibitor of protein synthesis to block antizyme synthesis therefore permits the unregulated uptake of polyamines. In the experiments presented here, we show that it also permits the very rapid accumulation of polyamine analogues to toxic levels and that this accumulation causes apoptosis within a short time period.

## EXPERIMENTAL

### Materials

The bis(ethyl) polyamine analogues used in these studies were generously provided by Dr. R. J. Bergeron (College of Pharmacy, University of Florida, Gainesville, FL, U.S.A.) {3,7,11,15-tetra-azaheptadecane [BE-3-3-3; also known as  $N^1, N^{11}$ -bis(ethyl)-norspermine], 3,7,12,16-tetra-azaoctadecane [BE-3-4-3, also known as  $N^1, N^{12}$ -bis(ethyl)spermine], and 3,8,13,18-tetra-aza-eicosane (BE-4-4-4)}, Dr. H. S. Basu (Department of Human Oncology, Medical School, University of Wisconsin-Madison, Madison, WI, U.S.A.) [1,19-bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4)], and Merrell Dow Research Institute (Cincinnati, OH, U.S.A.) { $N, N'$ -bis[3-(ethylamino)-propyl]-1,7-heptane diamine (BE-3-7-3)}.  $\alpha$ -Difluoromethylornithine (DFMO) and  $N^1$ -methyl- $N^2$ -(2,3-butadienyl)butane-1,4-diamine (MDL 72,527) were also obtained from Merrell Dow. Methylated polyamine derivatives, 1-methylspermidine (1-Me-SPD) and 1,12-dimethylspermine (1,12-Me<sub>2</sub>-SPM) [24] were kindly provided by Dr. J. K. Coward (Department of Chemistry, University of Michigan, Ann Arbor, MI, U.S.A.). *N*-Benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was purchased from Enzyme Systems (Dublin, CA, U.S.A.). SDS, Tris, 1-tosylamido-2-phenylethylchloromethyl ketone (TLCK), the cell death detection ELISA kit and proteinase K were obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Other chemicals and biomedical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Fisher Scientific (Fair Lawn, NJ, U.S.A.), Bio-Rad (Richmond, CA, U.S.A.), Gibco-BRL (Gaithersburg, MD, U.S.A.) and Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

### Cell culture and drug treatment

Chinese hamster ovary cells were grown as previously described in  $\alpha$ -minimum essential medium supplemented with 10% fetal bovine serum, 100  $\mu$ g/ml each penicillin and streptomycin [25]. Cells were grown at 37 °C with 5% CO<sub>2</sub> and 92% relative humidity. For each experiment, cells were plated in 60-mm tissue culture dishes with 5 ml of medium at a density of 10<sup>5</sup> cells per dish, unless DFMO was used. When used, 5 mM DFMO was added to the cell culture medium 4 h after cell plating at a density of 2 × 10<sup>5</sup> per dish. Cells were allowed to grow at 37 °C for 3 days. Polyamine analogues were then added to the culture medium in the presence or absence of 200  $\mu$ M cycloheximide and

the cells were incubated for a further 0–24 h as indicated. For experiments to test the effect of polyamine oxidase inhibitors and caspase inhibitors, cells were pre-treated with either 50  $\mu$ M of MDL 72,527 for 24 h or with 50  $\mu$ M of Z-VAD-fmk for 1.5 h before the addition of polyamine analogues.

### Analysis of DNA degradation and apoptosis

Production of cytoplasmic histone-associated DNA fragments, which is correlated to development of apoptosis, was analysed using the cell death detection ELISA kit (Boehringer-Mannheim GmbH, Germany) according to the manufacturer's instructions. Briefly, following cell culture, the attached cells were washed with two aliquots of 5 ml of serum-free medium, harvested with a disposable cell scraper, counted using a haemocytometer, and lysed with an appropriate amount of a buffer containing 1 mM EDTA/1% (w/v) BSA/0.2% (v/v) Tween-20 in PBS, pH 7, to obtain a lysate equivalent to 2 × 10<sup>6</sup> cells/ml. For each ELISA analysis, 0.01 ml of this lysate was used.

In order to measure the formation of DNA fragmentation ladders, cell extracts were prepared and analysed by agarose gel electrophoresis. The treated cells were washed twice with ice-cold PBS, resuspended with 35  $\mu$ l per 2 × 10<sup>6</sup> cells of lysis buffer (50 mM Tris/HCl, pH 9.0/20 mM EDTA/10 mM NaCl/1% SDS/1 mg/ml proteinase K) and digested overnight at 48 °C. The DNA was then extracted twice with phenol/chloroform (pre-equilibrated with a buffer containing 50 mM Tris/HCl, pH 9.0, 20 mM EDTA and 10 mM NaCl) and subsequently extracted once with chloroform/isoamyl alcohol (24:1). Each DNA sample (equivalent to the extract from 10<sup>6</sup> cells) was treated with a 1/100 volume of RNase A (10 mg/ml, heat-treated) and heated at 65 °C for 10 min. The samples were then separated by electrophoresis in 1.2% agarose gels using Tris/borate buffer (pH 8.0) containing 50  $\mu$ g/ml of ethidium bromide at 70–80 V for approximately 3 h. After electrophoresis, the DNA fragments were visualized and photographed under UV light.

### Analysis of polyamine analogue concentration

In order to assay the intracellular concentration of polyamine analogues, cell extracts were prepared, the polyamines converted into dansyl derivatives and the dansyl derivatives separated by HPLC and quantified using a fluorescence detector. After exposure to the polyamine analogues for the times indicated, the cells in each individual dish were washed three times in ice-cold PBS, harvested using a disposable cell scraper and resuspended in 0.1 ml of harvesting buffer containing 10 mM Tris, pH 7.5, 2.5 mM dithiothreitol and 0.1 mM EDTA. Then, 0.1 ml of 1.2 M perchloric acid was added and the protein precipitated was at 4 °C for at least 4 h. After centrifugation at 2000 rev./min (800 g) for 20 min, the supernatant containing the polyamines was removed. After the addition of 1,7-diaminoheptane, which was used as an internal standard, the samples were treated with dansyl chloride [25,26]. The dansyl derivatives were isolated and separated by reverse-phase HPLC on a Beckman (Fullerton, CA, U.S.A.) 5  $\mu$ m Ultrasphere ODS column (4.6 mm × 25 cm) at a flow rate of 2.5 ml/min at 50 °C using a gradient of acetonitrile/water running from 45:55 to 90:10 (v/v). Results were expressed as nmol polyamine/mg of cell protein. The cell protein was determined using the pellets from the perchloric acid precipitation step which were dissolved in 0.1 M NaOH and the protein content measured [27] using BSA as standard. The amount of Chinese hamster ovary cell protein per dish was between 95 and 125  $\mu$ g in all cases.

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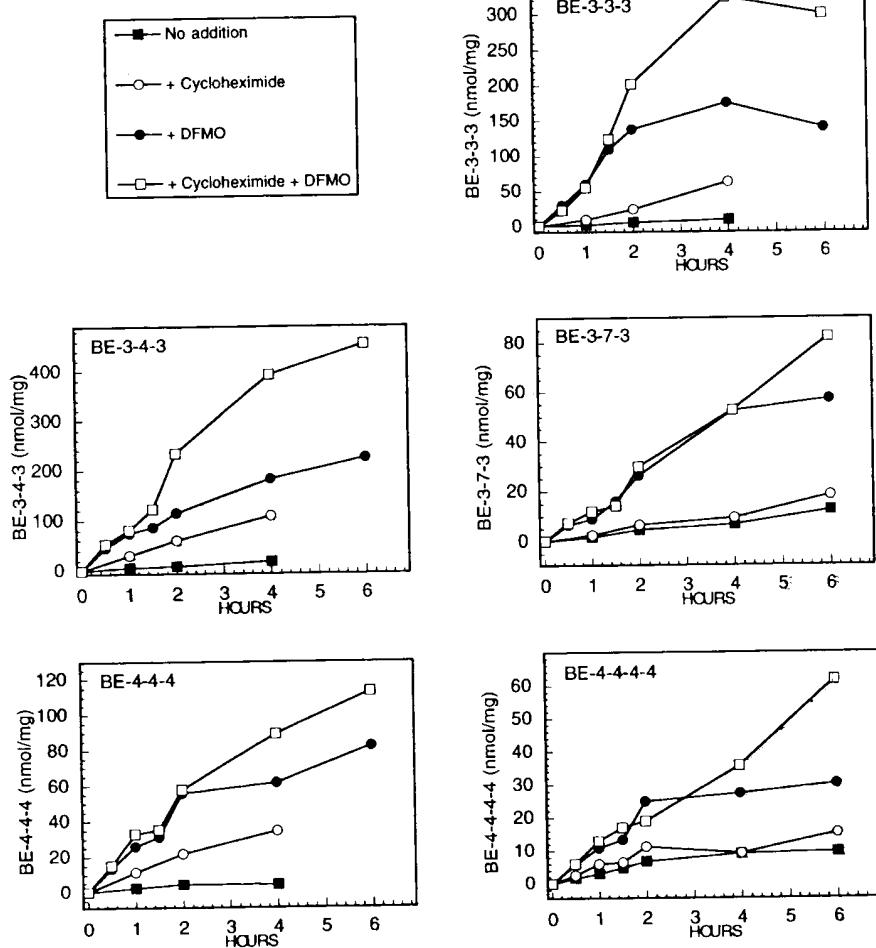
## RESULTS

### Effect of DFMO and cycloheximide on uptake of polyamine analogues

The uptake of bis(ethyl) polyamine analogues which have ethyl groups placed on the terminal nitrogen atoms [13,28] was studied by adding them at 10  $\mu$ M concentrations to the cell culture medium and measuring the intracellular concentration over the next 6 h. In otherwise untreated cells, there was a relatively slow uptake of these compounds, such that they reached values of 5.7–15.1 nmol/mg of protein within a 4 h period, with BE-3-3-3 being accumulated to the highest level and BE-4-4-4 to the lowest (Figure 1, filled square symbols). The uptake of all of the analogues except BE-3-7-3 and BE-4-4-4-4 was increased by adding cycloheximide, an inhibitor of protein synthesis, at the time of addition of the analogues (Figure 1, open circle symbols). Uptake of all analogues was increased greatly by pretreating the cells for 3 days before analogue addition with DFMO, which blocks endogenous polyamine synthesis and reduces cellular spermidine and putrescine to undetectable levels (Figure 1, filled circle symbols). The combination of DFMO and cycloheximide

had an even greater effect in increasing analogue uptake, particularly for BE-3-3-3 and BE-3-4-3 (Figure 1, open square symbols). Under these conditions, after 4 h of exposure to BE-3-3-3 or BE-3-4-3, the cells had accumulated 323  $\pm$  39 and 394  $\pm$  111 nmol of analogue/mg of protein respectively. This is vastly in excess of normal polyamine levels, which are about 15 nmol/mg of protein for both spermidine and spermine. Although the enhanced uptake of the other analogues was lower, it was still substantial. Within 6 h, the cells had accumulated 87  $\pm$  12 nmol/mg of BE-3-7-3, 111  $\pm$  7 nmol/mg of BE-4-4-4 and 61  $\pm$  9 nmol/mg of BE-4-4-4-4 (Figure 1).

It should be noted that in the cells in which repression of polyamine uptake was abolished by the use of DFMO or DFMO plus cycloheximide, the uptake of the analogues within a few hours was so great that a substantial reduction in the extracellular concentration in the medium was achieved. As shown in Table 1, as much as 74% of the total BE-3-3-3 and BE-3-4-3 added was present in the cells after 4–6 h of exposure to a starting extracellular concentration of 10  $\mu$ M. Even with the assumption that the extent of intracellular metabolism of the polyamine analogues is negligible, the extracellular concentration would therefore be



**Figure 1** Time course of accumulation of polyamine analogues

The intracellular content of polyamine analogue was determined as described in the Materials and methods section over a 6 h time period of exposure. Results are shown for the accumulation of 10  $\mu$ M BE-3-3-3, BE-3-4-3, BE-3-7-3, BE-4-4-4 and BE-4-4-4-4 as indicated. The cells were either untreated (■), treated with 200  $\mu$ M cycloheximide at the time of addition of the polyamine (○), treated with 5 mM DFMO for 3 days before the addition of the polyamine (●) or treated with both cycloheximide and DFMO (□). The intracellular analogue concentration was then determined as shown. Results are the mean of at least three observations that agreed within  $\pm$  10%.

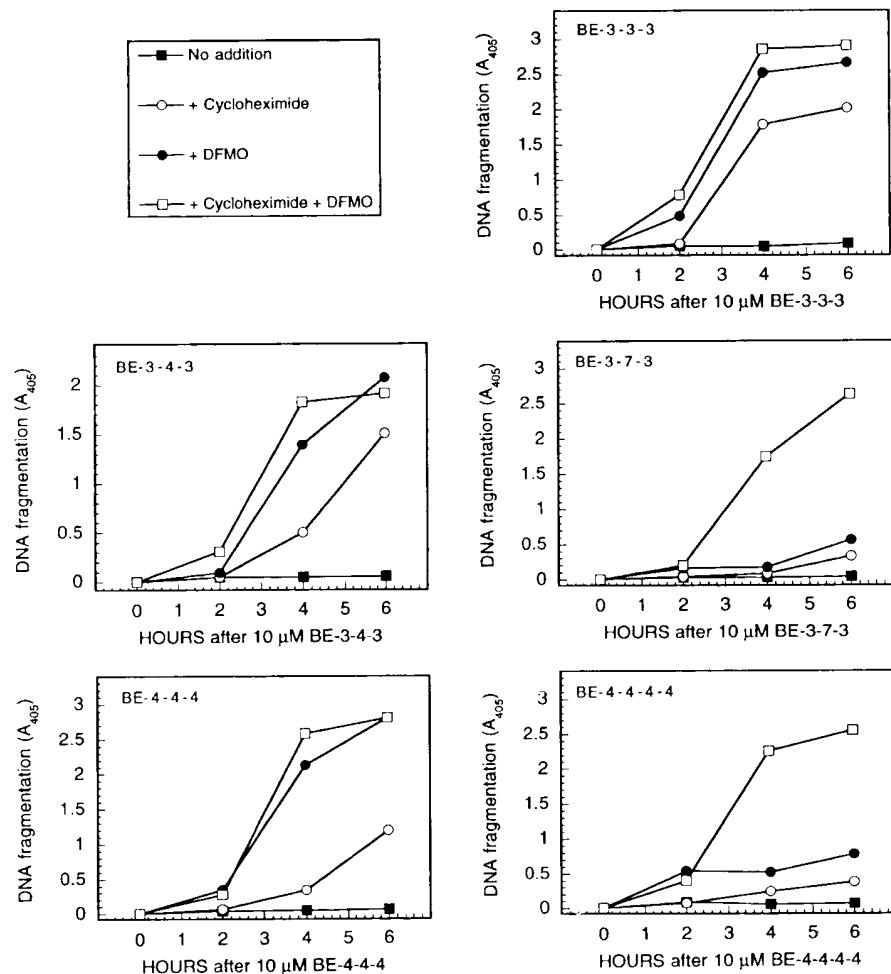
**Table 1 Total accumulation of polyamine analogues into Chinese hamster ovary cells treated with cycloheximide and DFMO**

Cells were exposed to the polyamine analogues as described in the legends to Figures 1, 3 and 8, and the total amount of analogue found in the cell is expressed as a percentage of the total added to the culture medium. ND, not determined.

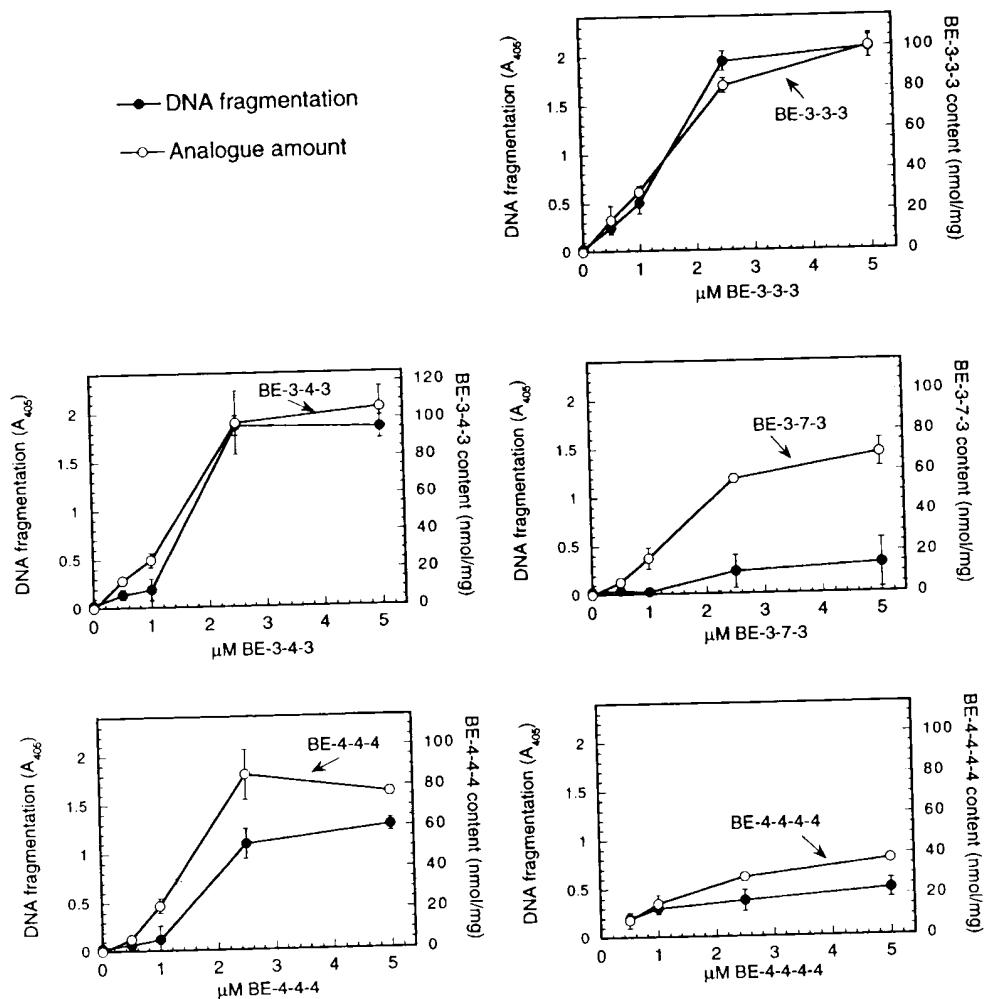
Treatment	Percentage of total added polyamine analogue present in cell pellet						
	BE-3-3-3	BE-3-4-3	BE-3-7-3	BE-4-4-4	BE-4-4-4-4	1-Me-SPD	1,12-Me <sub>2</sub> -SPM
10 $\mu$ M for 2 h	49	54	7	9	6	ND	ND
10 $\mu$ M for 3 h	ND	ND	ND	ND	ND	24	9
10 $\mu$ M for 4 h	74	66	10	12	9	ND	ND
10 $\mu$ M for 6 h	69	74	17	22	13	41	12
0.5 $\mu$ M for 4 h	70 <sup>a</sup>	57 <sup>a</sup>	28 <sup>b</sup>	26 <sup>b</sup>	31 <sup>b</sup>	ND	ND
1 $\mu$ M for 4 or 6 h	63 <sup>a</sup>	47 <sup>a</sup>	40 <sup>b</sup>	50 <sup>b</sup>	32 <sup>b</sup>	ND	ND
2.5 $\mu$ M for 4 or 6 h	70 <sup>a</sup>	81 <sup>a</sup>	46 <sup>b</sup>	67 <sup>b</sup>	26 <sup>b</sup>	ND	ND
5 $\mu$ M for 4 or 6 h	44 <sup>a</sup>	42 <sup>a</sup>	28 <sup>b</sup>	34 <sup>b</sup>	16 <sup>b</sup>	ND	ND

<sup>a</sup> Determined at 4 h.

<sup>b</sup> Determined at 6 h.

**Figure 2 Time course of induction of DNA fragmentation by polyamine analogues**

The extent of DNA fragmentation in response to polyamine analogues was determined as described in the Materials and methods section over a 6 h time period of exposure. Results are shown for BE-3-3-3, BE-3-4-3, BE-3-7-3, BE-4-4-4 and BE-4-4-4-4 as in Figure 1. Results are the means of at least three observations that agreed within  $\pm 10\%$ .



**Figure 3** Effect of polyamine analogue concentration on analogue accumulation and DNA fragmentation

Cells were treated with both DFMO and cycloheximide and measurements of analogue accumulation (○) and DNA fragmentation (●) were carried out as described in the legends to Figures 1 and 2. Results are shown for cells exposed to 0.5–5 μM BE-3-3-3 or BE-3-4-3 for 4 h, or to 0.5–5 μM BE-3-7-3, BE-4-4-4 or BE-4-4-4-4 for 6 h as indicated. Results ± S.E.M. are given for at least four estimations.

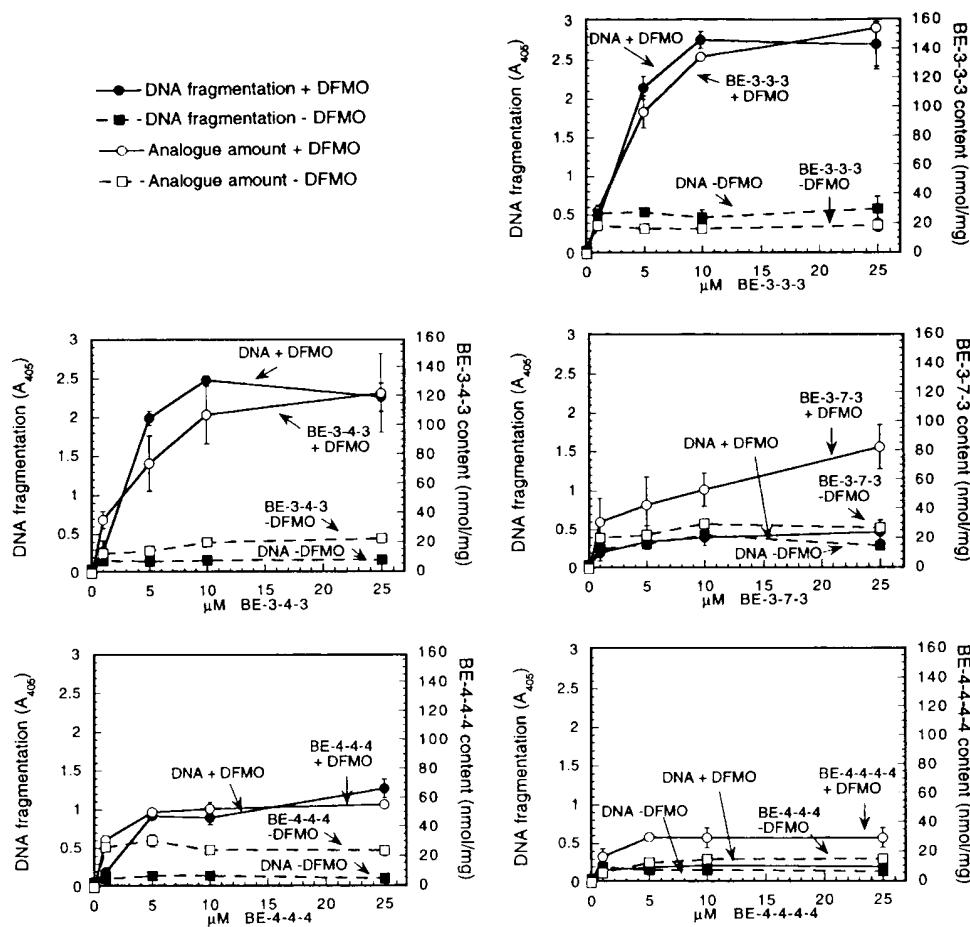
decreased to less than 3 μM. Therefore, it is possible that the reduction in the extracellular analogue concentration under these conditions may limit further uptake. This could reduce the differences between the uptake of BE-3-3-3 and BE-3-4-3 and the uptake of the other analogues which were accumulated to lower extents. Some support for this is seen when the uptake period was fixed at 4 h and the analogue concentration was varied. With starting extracellular concentrations of 2.5 μM or less, even BE-4-4-4-4 was accumulated to levels amounting to more than 25% of the total added. With exposure to 0.5–1 μM external concentrations, all of the analogues were accumulated in the cells to such an extent that 25–70% of the total added was taken up (Table 1).

#### Effect of analogue accumulation on cell death

These large accumulations of polyamine analogues had a rapid toxic effect on the cells. There was morphological evidence of apoptosis within 2–4 h of exposure to BE-3-3-3 and BE-3-4-3 and 4–6 h of exposure to the other analogues. Quantification of this effect was obtained using an ELISA method for the presence

of cytoplasmic nucleosomal degradation products (Figure 2). Such products were present at 4 h in cells treated with all of the analogues in the presence of DFMO and cycloheximide. When cycloheximide or DFMO were omitted, the DNA fragmentation was still seen in the cells treated with BE-3-3-3 and BE-3-4-3, and to a lesser extent with BE-4-4-4. These results correlate quite well with the accumulation of the analogues seen in Figure 1, and suggest that the high intracellular levels of polyamine analogues induce apoptosis and that BE-3-3-3 and BE-3-4-3 were more potent than the other analogues in causing such cell death.

This conclusion was supported by the results of experiments in which the concentration of the analogue added to the culture medium was varied up to 5 μM and the level of the analogue and the occurrence of DNA fragmentation were measured 4–6 h later (Figure 3). In this case, BE-3-3-3 and BE-3-4-3 were accumulated to a very high extent (Table 1), and maximal levels of cell death occurred in cells treated with 2.5 μM concentrations. Such exposure led to the intracellular accumulation of about 100 nmol of BE-3-3-3 or BE-3-4-3 per mg of protein. BE-4-4-4 was somewhat less active, but it only accumulated to about 80 nmol/mg of protein, and BE-3-7-3 and BE-4-4-4-4, which



**Figure 4** Effect of DFMO on analogue accumulation and DNA fragmentation

Control cells (□, ■) or cells treated with 5 mM DFMO for 3 days (○, ●) were exposed to 0–25 μM of BE-3-3-3, BE-3-4-3, BE-3-7-3, BE-4-4-4 or BE-4-4-4-4 for 6 h as indicated. Analogue accumulation (□, ○) and DNA fragmentation (■, ●) were measured as described in the legends to Figures 1 and 2. Results ± S.E.M. are given for at least four estimations.

accumulated to only 70 and 40 nmol/mg of protein respectively, were considerably less effective in causing cell death.

In all of the experiments shown in Figure 3, both DFMO and cycloheximide were added to maximize uptake of the analogues. As shown in Figure 4, when both of these compounds were omitted, there was very little induction of apoptosis with up to 25 μM of any of the analogues, but the analogue intracellular content was always below 40 nmol/mg of protein. When DFMO but not cycloheximide was used, maximal effects on apoptosis were induced by BE-3-3-3 and BE-3-4-3 and these correlated with intracellular levels of > 80 nmol/mg of protein (Figure 4). These results demonstrate clearly that the induction of apoptosis is not related to the inhibition of protein synthesis by cycloheximide, but to the accumulation of the polyamine analogues. In the presence of DFMO alone, BE-4-4-4 produced a significant but lower effect on cell killing and was accumulated to levels of around 50–60 nmol/mg (Figure 4). BE-3-7-3 and BE-4-4-4-4 were less effective within the 6 h period studied, even though the content of BE-3-7-3 did reach 80 μmol/mg of protein at exposures of 25 μM.

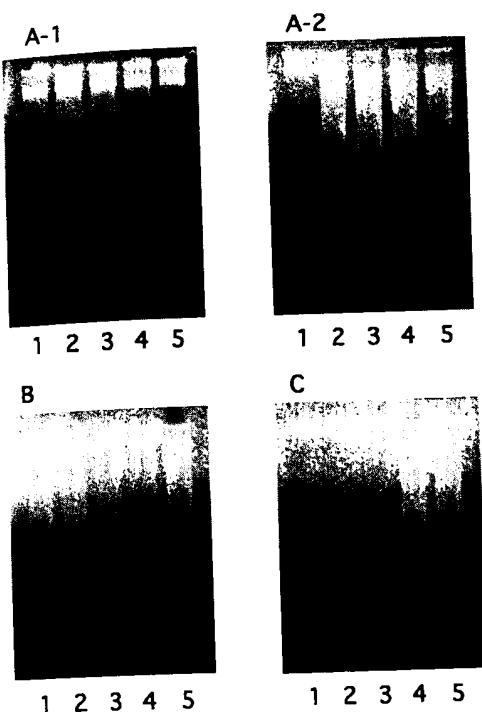
Further evidence for DNA fragmentation in response to polyamine analogues was obtained by studies in which DNA fragments were separated on agarose gels (Figure 5). A time course of the results of exposure to 1 μM or 2.5 μM BE-3-3-3

showed clear evidence that DNA ladders, characteristic of apoptosis, were present in the cells 2–8 h after exposure to BE-3-3-3 (Figure 5, panels A1 and A2). Such ladders were also observed in cells treated with BE-3-3-3, BE-3-4-3, BE-4-4-4, BE-3-7-3 and BE-4-4-4-4 (Figure 5, panel B). These results show that BE-3-7-3 and BE-4-4-4-4 do produce programmed cell death within a short time period, even though the incidence was too low to be measured easily with ELISA used in the experiments shown in Figure 4.

Although exposure to cycloheximide increased the potency with which BE-3-3-3 induced ladder formation, it was not necessary for this effect, since the characteristic pattern of DNA fragmentation was readily apparent in cells treated for 4 h with 5 and 10 μM BE-3-3-3 without any exposure to cycloheximide (Figure 5, panel C).

#### Effect of a caspase inhibitor on apoptosis in response to polyamine analogues

Production of DNA fragmentation ladders (Figure 6, panels A and B) and the appearance of cytoplasmic nucleosomal fragments (Figure 6, panel C) in response to BE-3-3-3 was blocked by the caspase inactivator Z-VAD-fmk, suggesting that the activity of



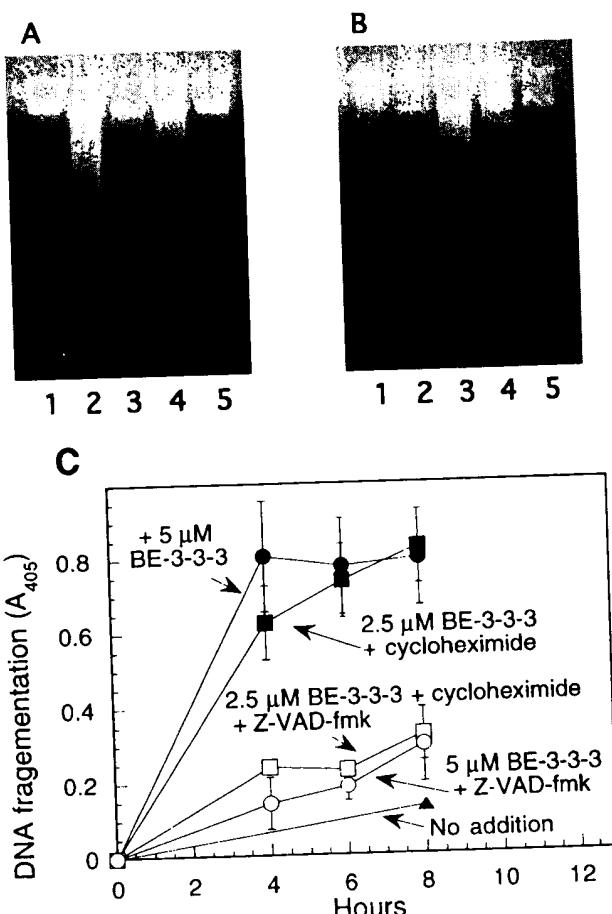
**Figure 5** Degradation of nuclear DNA by polyamine analogues

Cells were treated and harvested and DNA was isolated and analysed by agarose gel electrophoresis for the production of oligodeoxynucleotide ladders, as described in the Materials and methods section. In (A-1) and (A-2), results are shown for cells treated with no addition or 5  $\mu$ M addition of either 1 or 2.5  $\mu$ M BE-3-3-3 for 2–12 h. Results are for cells treated with 5 mM DFMO and 200  $\mu$ M cycloheximide, as described in the legend to Figure 1, and then exposed to either (A-1) 1  $\mu$ M BE-3-3-3 for 2 h (lane 1), 4 h (lane 2), 6 h (lane 3), 8 h (lane 4) and 12 h (lane 5); or to (A-2) 2.5  $\mu$ M BE-3-3-3 for 2 h (lane 1), 4 h (lane 2), 6 h (lane 3), 8 h (lane 4) and 12 h (lane 5). Lanes 1 in (A-1) and (A-2) show results for no addition. Results in (B) are for cells treated with 5 mM DFMO and 200  $\mu$ M cycloheximide and then incubated for 4 h with 2.5  $\mu$ M BE-3-3-3 (lane 1), 2.5  $\mu$ M BE-3-4-3 (lane 2), 2.5  $\mu$ M BE-4-4-4 (lane 3), 5  $\mu$ M with 2.5  $\mu$ M BE-3-3-3 (lane 4), and 5  $\mu$ M BE-4-4-4 (lane 5). Results in (C) are for cells treated with 5 mM BE-3-3-3, and then exposed to BE-3-3-3 for 4 h. DFMO alone, as described in the legend to Figure 1, and then exposed to BE-3-3-3 for 4 h. The BE-3-3-3 concentrations used were: 0  $\mu$ M (lane 1), 1  $\mu$ M (lane 2), 2.5  $\mu$ M (lane 3), 5  $\mu$ M (lane 4) and 10  $\mu$ M (lane 5).

a caspase that is sensitive to this inhibitor [29] is essential for the induction of apoptosis. This inhibition occurred in cells treated with 5  $\mu$ M BE-3-3-3 or with cells treated with 2.5  $\mu$ M BE-3-3-3 in the presence of cycloheximide. The presence of Z-VAD-fmk had no effect on the intracellular accumulation of BE-3-3-3, which after exposure to 2.5  $\mu$ M for 4 h in the presence of cycloheximide was  $82.2 \pm 2.6$  nmol/mg of protein in the presence of Z-VAD-fmk and  $83.9 \pm 10.9$  in its absence. In the absence of cycloheximide, treatment with 5  $\mu$ M BE-3-4-3 led to accumulation of  $59.6 \pm 8.0$  nmol/mg of protein in the presence of Z-VAD-fmk and  $58.5 \pm 5.1$  in its absence.

#### Effect of MDL 72,527, a polyamine oxidase inhibitor, on apoptosis and on polyamine metabolism

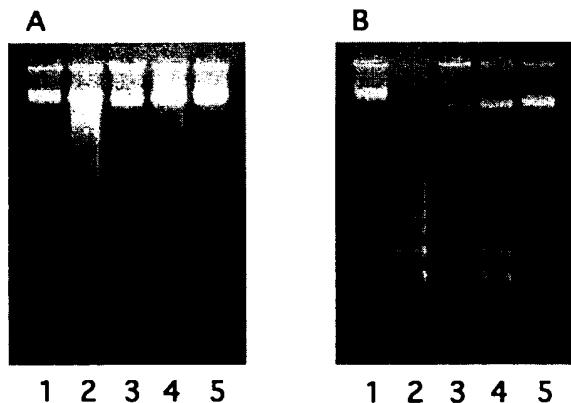
MDL 72,527 has been reported to be a potent and specific inhibitor of polyamine oxidase [30,31]. As shown in lane 5 of Figure 6(A), and in Figure 7(A), the production of DNA fragmentation ladders typical of apoptosis in cells treated with BE-3-3-3, BE-3-4-3 or BE-3-7-3 was blocked by the presence of MDL 72,527. MDL 72,527 also effectively prevented apoptosis in response to BE-4-4-4 for 4 h or BE-4-4-4 for 6 h (results not



**Figure 6** Effect of inhibitors on induction of apoptosis by polyamine analogues

Cells were treated with the potential inhibitors, used for 90 min before exposure to polyamine analogues, and DNA analysis was carried out as described in the legend to Figure 5. The results are shown in (A) and (B). DNA fragmentation was also carried out as described in the legend to Figure 3 and the results are shown in (C). (A) Samples from cells treated with DFMO and then pretreated with caspase inhibitor for 90 min or with PAO inhibitor, 24 h before exposure to 5  $\mu$ M BE-3-3-3 for 4 h. Results are shown for no treatment (lane 1), 5  $\mu$ M BE-3-3-3 (lane 2), 5  $\mu$ M BE-3-3-3 + 50  $\mu$ M Z-VAD-fmk (lane 3), 5  $\mu$ M BE-3-3-3 + 100  $\mu$ M TLCK (lane 4); and 5  $\mu$ M BE-3-3-3 + 50  $\mu$ M MDL 72,527 (lane 5). (B) Samples from cells treated with: 5 mM DFMO (72 h) and 50  $\mu$ M Z-VAD-fmk (90 min) and exposed to 5  $\mu$ M BE-3-3-3 for 4 h (lane 1), 5 mM DFMO (72 h), 200  $\mu$ M cycloheximide (4 h) and 50  $\mu$ M Z-VAD-fmk (90 min) and exposed to 2.5  $\mu$ M BE-3-3-3 for 4 h (lane 2); 5 mM DFMO (72 h) and exposed to 5  $\mu$ M BE-3-3-3 for 4 h (lane 3); 5 mM DFMO (72 h) and 200  $\mu$ M cycloheximide (4 h) and exposed to 2.5  $\mu$ M BE-3-3-3 for 4 h (lane 4); 5 mM DFMO (72 h) alone (lane 5). (C) Time course of DNA fragmentation in cells treated with 5 mM DFMO for 72 h and exposed to 5  $\mu$ M BE-3-3-3 (○), or with 5 mM DFMO and 200  $\mu$ M cycloheximide (□), and exposed to 2.5  $\mu$ M BE-3-3-3 in the presence (○, □) or absence of Z-VAD-fmk treatment (■, ▲).

shown). In contrast, MDL 72,527 had no effect on the production of apoptotic DNA ladders by staurosporine (Figure 7B). Treatment with MDL 72,527 did lead to an increase in the content of N<sup>1</sup>-acetylspermidine in control cells treated with 10  $\mu$ M BE-3-4-3 or BE-3-7-3 (results not shown), but it had no statistically significant effect on the content of the polyamine analogues, which were  $49.2 \pm 2.0$  nmol/mg in the presence of MDL 72,527 compared with  $45.1 \pm 5.5$  nmol/mg for BE-3-4-3, and  $39.7 \pm 8.0$  nmol/mg in the presence of MDL 72,527 compared with  $36.5 \pm 5.7$  for BE-3-7-3. The same lack of effect on analogue accumulation was found when 50  $\mu$ M MDL 72,527 was added to cells treated with DFMO for 3 days and with BE-3-4-3 for 4 h or



**Figure 7 Effect of MDL 72,527 on induction of apoptosis by polyamine analogues**

Cells were treated and harvested and the DNA was isolated and analysed by agarose gel electrophoresis for the production of oligodeoxynucleotide ladders, as described in Figure 5. (A) Cells treated with 50  $\mu$ M MDL 72,527 added 24 h before the addition of BE-3-3-3 and harvested 4 h later. Results are shown for cells treated with: 5 mM DFMO (72 h) alone (lane 1); 5 mM DFMO (72 h) and exposed to 5  $\mu$ M BE-3-3-3 for 4 h (lane 2); 5 mM DFMO (72 h), 50  $\mu$ M MDL 72,527 (24 h) and exposed to 5  $\mu$ M BE-3-3-3 for 4 h (lane 3); 5 mM DFMO (72 h) and 200  $\mu$ M cycloheximide and exposed to 2.5  $\mu$ M BE-3-3-3 for 4 h (lane 4); 5 mM DFMO (72 h), 200  $\mu$ M cycloheximide, 50  $\mu$ M MDL 72,527 (24 h) and exposed to 2.5  $\mu$ M BE-3-3-3 for 4 h (lane 5). (B) Cells treated with: no addition (lane 1); 2  $\mu$ M staurosporine for 12 h (lane 2); 2  $\mu$ M staurosporine for 12 h + 50  $\mu$ M MDL 72,527 for 24 h (lane 3); 2  $\mu$ M staurosporine and 200  $\mu$ M cycloheximide for 12 h (lane 4); 2  $\mu$ M staurosporine and 200  $\mu$ M cycloheximide for 12 h + 50  $\mu$ M MDL 72,527 for 24 h (lane 5).

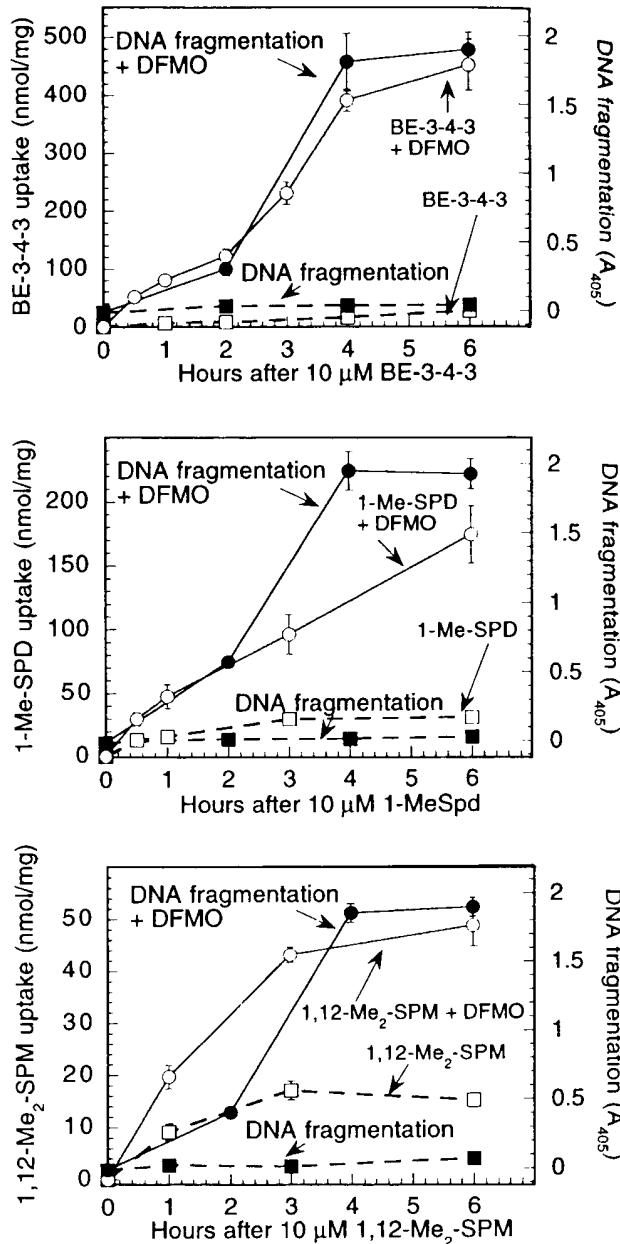
BE-3-7-3 for 4 h. In these cells, all of the polyamines, except spermine, were reduced to below the limit of detection and the BE-3-4-3 content was unchanged at  $69.1 \pm 9.5$  nmol/mg of protein in the absence of MDL 72,527 and  $72.4 \pm 6.5$  in its presence. The corresponding values for BE-3-7-3-treated cells were  $59.0 \pm 17.4$  and  $79.0 \pm 19.8$  nmol/mg.

#### Comparison of N-terminally and C-terminally alkylated polyamine analogues

The rapid induction of apoptosis in cells with a deregulated polyamine transport system was not limited to the bis(ethyl) polyamine analogues with substitutions on the terminal nitrogen atoms. As shown in Figure 8, both 1-Me-SPD and 1,12-Me<sub>2</sub>-SPM, in which the methyl groups are placed on the terminal carbon atoms in the polyamine backbone [24], were also able to induce this effect within 4–6 h after exposure to 10  $\mu$ M concentrations. All three compounds produced identical effects on cell killing, and since 1,12-Me<sub>2</sub>-SPM was accumulated to levels of only about 50 nmol/mg of protein, this analogue was the most active if considered in terms of the effective intracellular concentration.

#### DISCUSSION

These results show clearly that the rapid cellular accumulation of polyamine analogues to high levels causes apoptotic cell death. This conclusion is in agreement with the report that exposure of breast cancer cells to an unsymmetrical polyamine analogue for prolonged periods (but without manipulation to increase analogue uptake) led to programmed cell death [14]. Although the most effective conditions for producing such accumulation in our experiments involved the use of the protein synthesis inhibitor cycloheximide, to prevent antizyme-mediated repression of poly-



**Figure 8 Comparison of DNA fragmentation and analogue incorporation between polyamine analogues having N-ethyl and C-methyl substituents**

Experiments were carried out as described in the legend to Figure 3. Cells were treated with both DFMO and cycloheximide and exposed to 10  $\mu$ M BE-3-4-3 (upper panel), 10  $\mu$ M 1-Me-SPD (middle panel) or 10  $\mu$ M 1,12-Me<sub>2</sub>-SPM (lower panel). Control cells (□, ■) or cells treated for 3 days with DFMO (○, ●) were exposed to 200  $\mu$ M cycloheximide and 10  $\mu$ M BE-3-3-3, 1-Me-SPD or 1,12-Me<sub>2</sub>-SPM as indicated. Analogue accumulation (□, ○) and DNA fragmentation (■, ●) were measured at 1–6 h after analogue addition, as shown. Results  $\pm$  S.E.M. are given for at least four estimations.

amine transport, the results shown in Figures 4 and 5 provide convincing evidence that BE-3-3-3 and BE-3-4-3 can cause apoptosis without cycloheximide treatment. These are the two analogues that are accumulated to the highest levels and, even without cycloheximide, reached values of more than 150 nmol/mg of protein within 4 h. However, all of the analogues tested were able to induce apoptosis, as indicated by the more

sensitive test of the appearance of DNA fragmentation ladders shown in Figure 5(B). Further studies will be needed to examine the mechanism by which apoptosis is induced in response to polyamine analogues, but the system described here, in which a profound apoptotic effect occurs within 2 h of exposure to 2.5  $\mu$ M BE-3-3-3 (see Figure 5A-2, lane 2), provides a useful experimental system to investigate this further.

It is possible that the depletion of normal intracellular polyamines facilitates cell killing by the analogues in ways other than permitting increased uptake, for example, by allowing binding to sites normally occupied by spermidine or spermine, but it appears more likely that it is merely the high intracellular level of the analogues that brings about the effect. As shown in Figure 8, even the C-terminally methylated polyamines, which are much more similar to the natural counterparts than BE-3-4-3, were able to cause apoptosis. In fact, these compounds were more potent than the bis(ethyl) analogues if expressed in terms of the intracellular level associated with toxicity. It is therefore likely that cells are unable to survive with highly elevated levels of polyamines themselves or of the polyamine analogues.

This suggestion is consistent with a number of preliminary reports suggesting that accumulation of high levels of the normal polyamines can cause apoptotic cell death. Thus, overproduction of polyamines resulting from c-myc-mediated over-expression of ODC [32] led to apoptosis. Similarly, accumulation of spermidine in L1210 cells with an amplification of the ODC gene caused apoptosis [11]. These cells have a polyamine transport system that is not stringently repressed by polyamines, probably due to the binding of all the available antizyme to the excess ODC [33]. When such ODC-overproducing L1210 cells were exposed to a hypotonic shock, the transport of exogenous spermidine was greatly increased and, because of the absence of feed-back repression, spermidine accumulated to toxic levels [11]. Uncontrolled uptake of spermidine into variant HTC cells with an elevated ODC content also led to toxicity and cell death, although the mechanism of killing was not investigated [34]. There is experimental evidence that even putrescine, which is usually thought of as a precursor of polyamines, can be accumulated to levels that cause apoptotic cell death when cells that greatly overproduce ODC are also provided with an excess of the ornithine substrate [35,36]. These studies provide support for the contention that the powerful homoeostatic regulation of the polyamine biosynthetic pathway, which is mediated by effects on both the synthesis and degradation of key enzymes in polyamine synthesis and excretion [1,2], may be essential to prevent the accumulation of polyamines to highly toxic levels [5] unless sequestration of the polyamines can take place [6].

It is well known that Bcl-2 is a key protein in protecting cells from apoptosis, and recent studies have shown that this protein, which is associated with mitochondrial and nuclear membranes, has a structure resembling pore-forming domains of bacterial toxins and may form an ion channel [37]. Polyamines and analogues have been shown to have the ability to block or modulate ion channels such as the N-methyl-D-aspartate receptor and the strong inward rectifier K<sup>+</sup> channels [38,39]. These results raise the intriguing possibility that interaction with a Bcl-2-mediated ion channel may be responsible for the toxicity of excess polyamines or analogues. It is also noteworthy that the disruption of the mitochondrial transmembrane potential and release of cytochrome c has been implicated as an early stage in apoptosis that precedes nuclear fragmentation and is prevented by Bcl-2 [40,41]. Mitochondrial accumulation of polyamines occurs very rapidly, and previous studies have related cell killing by polyamines or analogues to mitochondrial effects [19,42].

The ability of the polyamine analogues to cause apoptosis was

antagonized by treatment with MDL 72,527, which is well established as a specific inhibitor of the PAO [30,31]. It therefore appears that PAO activity may be needed for the toxic effects. The physiological substrates for PAO are N<sup>1</sup>-acetylspermine and N<sup>1</sup>-acetylspermidine, which are converted into 3-acetamido-propanal, hydrogen peroxide and spermine or spermidine respectively. PAO is therefore thought to be involved in the interconversion and degradation of polyamines. However, PAO is also able to attack N-alkyl- $\alpha,\omega$ -diaminoalkanes, producing an aldehyde, hydrogen peroxide and the free analogue [43,44], and is likely to play a role in the metabolism of the bis(ethyl) analogues. Mono-dealkylated species consistent with such a reaction have been observed in animals treated with BE-3-3-3 [45] and BE-4-4-4 [46]. Although the extent of such de-ethylation is likely to be small, since MDL 72,527 had no statistically significant effect on the levels of BE-3-4-3 or BE-3-7-3, the possibility that sufficient amounts of such metabolites accumulate to initiate apoptosis cannot be ruled out. However, a more likely explanation is that the hydrogen peroxide or other reactive oxygen species generated in the PAO reaction are involved in the toxicity. The induction of programmed cell death in response to reactive oxygen species is well known and the prevention of such effects is a function of Bcl-2 [47]. Both oxidation-dependent [48,49] and -independent mechanisms for the induction of apoptosis by spermidine have been described [11].

The levels of polyamine analogues reached in the cells under the experimental conditions described in the current work far exceed those likely to occur in normal growth and development. However, these levels lead to programmed cell death in all of the cells within a very short period of time. Our results raise the suggestions that: (1) elevated polyamine levels may contribute to apoptosis in response to both exogenous and physiological endogenous stimuli, possibly via the formation of toxic oxidation products [8]; and (2) that the antitumour effects of polyamine analogues may be mediated via the induction of apoptosis in tumour cell types that respond differentially to exposure to polyamine analogues [14].

This research was supported by grant GM-26290 from the National Institutes of Health.

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Received 12 May 1997/22 July 1997; accepted 11 August 1997